Spectroscopic and Chemical Studies of the Ascorbate Oxidase Trinuclear Copper Active Site: Comparison to Laccase

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Abstract: The multicopper oxidases (laccase, ascorbate oxidase, and ceruloplasmin) contain type 1, 2, and 3 copper sites. Detailed spectroscopic studies of azide binding to laccase have demonstrated that the type 2 and the coupled binuclear type 3 centers form a trinuclear Cu cluster site (Allendorf, M. D.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3063-3067) that has been shown to be the active site in the multielectron reduction of dioxygen (Cole, J. L.; et al. *J. Am. Chem. Soc.* **1990**, *112*, 2243-2249). A recent X-ray crystal structure of ascorbate oxidase indicates that this enzyme also contains a trinuclear site (Messerschmidt, A.; et al. *J. Mol. Biol.* **1989**, *206*, 513-529). In the present study, a combination of electronic spectroscopy and exogenous ligand perturbation has been used to probe the geometric and electronic structures of the type 1 and the type 2-type 3 trinuclear sites in ascorbate oxidase. These results are compared to our previous work on laccase. The low-temperature magnetic circular dichroism (MCD) spectra of the type 1 centers in ascorbate oxidase and plastocyanin are very similar, but the type 1 spectrum of laccase is different, indicating that the structure of the laccase blue copper center is perturbed. The contribution to the MCD spectrum by the type 2 Cu^{2+} is identified by the effect of fluoride binding to the type 2 site, and the energies closely correspond to the type 2 features in laccase. Azide equilibrium binding and kinetic measurements demonstrate that three different azide molecules coordinate to the trinuclear site. One azide binds as a bridging ligand between the type 2 site and the type 3 site in a manner that is similar to what we previously observed in laccase. In contrast, the two other azides bind terminally to the type 2 and type 3 coppers, respectively, whereas in laccase only a second azide binds to the fully oxidized enzyme and bridges the type 2 and type 3 sites. This difference indicates the presence of a distortion of the ascorbate oxidase trinuclear site that prevents an additional azide from bridging the type 2 and type 3 centers. The conservation of the type 2-type 3 bridged binding site for azide in the two enzymes suggests that this coordination mode is active in the irreversible four-electron reduction of dioxygen to water in the multicopper oxidases and that only a single bridging coordination position is required for efficient four-electron dioxygen reduction.

Introduction

The multicopper oxidases,[1] ascorbate oxidase, laccase, and ceruloplasmin, catalyze the four-electron reduction of dioxygen to water. The Cu atoms present in these enzymes have been classified[1] according to their EPR features: type 1 or blue ($A_{\parallel} \leq 95 \times 10^{-4}$ cm^{-1}), type 2 or normal ($A_{\parallel} > 140 \times 10^{-4}$ cm^{-1}), and type 3 or coupled binuclear (EPR nondetectable). The type 3 coppers are strongly antiferromagnetically coupled so that the type 3 site is diamagnetic, even at room temperature.[2,3] The superexchange interaction responsible for this antiferromagnetism is mediated by a bridging ligand, OR^-,[4] which appears to be OH^- on the basis of the ascorbate oxidase crystal structure.[5] The various copper centers also give rise to electronic features that have been well characterized in laccase.[3,6,8] The type 1 center exhibits an intense cys S $\rightarrow$ Cu^{2+} charge transfer (CT) absorption band near 600 nm ($\Delta\epsilon \sim 5000$ M^{-1} cm^{-1}) and several weaker features from the UV to the near-IR region.[4] The oxidized type 3 center displays a broad near-UV absorption band maximizing at 330 nm, $\Delta\epsilon = 2800$ M^{-1} cm^{-1},[6,7] and several d $\rightarrow$ d absorption and CD features from 585 to 900 nm.[3] The type 2 magnetic circular dichroism (MCD) spectrum consists of several weak CT bands from 300 to 400 nm and two d $\rightarrow$ d bands at 720 and 860 nm.[3]

Much of the research on the multicopper oxidases has focused on defining the structure and reactivity of the dioxygen reduction site. Laccase is the simplest multicopper oxidase, containing one type 1, one type 2, and one coupled binuclear type 3 center for a total of four copper atoms. We have demonstrated through low-temperature MCD studies that the exogenous anionic inhibitor N_3^- binds to laccase as a bridging ligand between the type 2 site and one of the type 3 coppers, thereby defining a novel trinuclear copper cluster.[3,8,9] In addition, a second azide coordinates to the type 3 site with a lower binding constant, and this second azide also likely binds in a bridging coordination mode.[3] It was suggested that this trinuclear site represents the true active site for the binding and multielectron reduction of dioxygen,[8] and we have recently demonstrated using Cu X-ray absorption edge measurements that this trinuclear Cu site is the minimum structural unit required for dioxygen reduction.[10] Ascorbate oxidase contains two subunits and two of each type of copper center.[11] Recently, a 2.5-Å X-ray structure of ascorbate oxidase has supported the trinuclear copper cluster model, showing that each subunit contains three copper atoms within 3.9 Å of each other and a type 1 Cu^{2+} site ~12 Å away from the trinuclear cluster.[5] However, in

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contrast to the case of laccase, the spectroscopic and chemical properties of the copper centers in ascorbate oxidase have not been studied in detail. The electronic and EPR spectral features of the type 1, 2, and 3 centers in ascorbate oxidase appear similar to the corresponding features in laccase.[12] In studies of azide binding to ascorbate oxidase, two $N_3^- \rightarrow Cu^{2+}$ CT bands have been reported at ~420 and 500 nm.[13] Similar $N_3^- \rightarrow Cu^{2+}$ CT features are observed in laccase,[3,8a,b] and it has been suggested that azide binds similarly in the two enzymes.[13d,e] However, the ascorbate oxidase results are complicated by the fact that more than one azide molecule binds (vide infra). Thus, direct spectroscopic and chemical evidence that azide bridges the type 2 and type 3 sites in ascorbate oxidase has not been established.

One difference between ascorbate oxidase and laccase concerns the efficiencies of various chelating agents in the removal of the type 2 copper. In ascorbate oxidase, type 2 copper is completely removed by treatment with EDTA, dimethylglyoxime, *N,N*-diethyldithiocarbamate, or nitrilotriacetate.[14] In contrast, the laccase type 2 copper can only be extracted by nitrilotriacetate or by EDTA in combination with the other chelators. It was concluded from these studies that the type 2 copper is less accessible in laccase than in ascorbate oxidase.[14]

Low-temperature MCD is extremely powerful in discriminating the paramagnetic type 1 and type 2 transitions from the electronic transitions of the antiferromagnetically coupled type 3 center in laccase,[3,8] and a similar approach is taken here for ascorbate oxidase. Briefly, the paramagnetic sites give rise to MCD bands whose intensities are proportional to $1/T$ for $kT \gg g\beta H$ (C terms),[15] while the MCD features associated with the magnetically nondegenerate $S_{tot} = 0$ ground state of the antiferromagnetically coupled type 3 center are temperature independent (A and B terms).[15] Near 4 K, the MCD features associated with the antiferromagnetically coupled type 3 center are 100–1000 times weaker than those from the paramagnetic type 1 and type 2 centers; therefore the MCD spectrum of ascorbate oxidase is dominated by contributions from the type 1 and type 2 centers.

In the present study, absorption, low-temperature MCD, and EPR spectroscopies and exogenous ligand binding are used to probe the electronic and geometric structure of both the type 1 center and the type 2–type 3 trinuclear site in native ascorbate oxidase. Fluoride binding produces characteristic changes in the type 2 Cu^{2+} MCD spectrum that allow us to assign the type 2 electronic features even in the presence of the type 1 site.[3] Azide binding gives rise to intense azide $\rightarrow Cu^{2+}$ CT bands and perturbs the Cu^{2+} d $\rightarrow$ d transitions. We find from thermodynamic and kinetic meaurements that three different azide molecules bind to the ascorbate oxidase trinuclear copper site. Their spectral features are determined and are used to define their binding geometries, which show coordination similarities and differences relative to exogenous ligand binding to the trinuclear site in laccase.

Materials and Methods

Ascorbate oxidase was purified from green zucchini as previously described.[16] The concentration was assayed using the absorption band at 610 nm. Ascorbate oxidase contains two trinuclear sites per dimeric unit, and to facilitate comparison with our previous work on laccase, the ascorbate oxidase spectra reported here are presented in terms of absorptivity per mole of *trinuclear sites* rather than per mole of enzyme (ϵ_{610} = 5000 M^{-1} cm^{-1}).[12a] Copper concentration was determined by atomic absorption spectroscopy. Samples for MCD spectroscopy were concentrated to ~0.25 mM using Immersible-CX ultrafilters (Millipore Corp.) or an Amicon ultrafiltration cell (Amicon Corp.) and were subsequently dialyzed against 50% (v/v) glycerol/0.2 M potassium phosphate pH 6.0 buffer, which provides a good-quality low-temperature glass. All spectroscopic measurements were performed at an enzyme concentration of ~0.6 mM. For ligand titration experiments, 5–10-μL portions of concentrated aqueous anion solutions were added to 150–200-μL aliquots of enzyme, and samples were allowed to equilibrate at least 24 h at 4 °C prior to spectroscopic measurements. Azide-binding constants were determined by direct nonlinear least-squares fitting to the titration data. All chemicals were reagent grade and were used without further purification. Water was purified to a resistivity of 16–18 MΩ cm using a Barnstead Nanopure deionizing system.

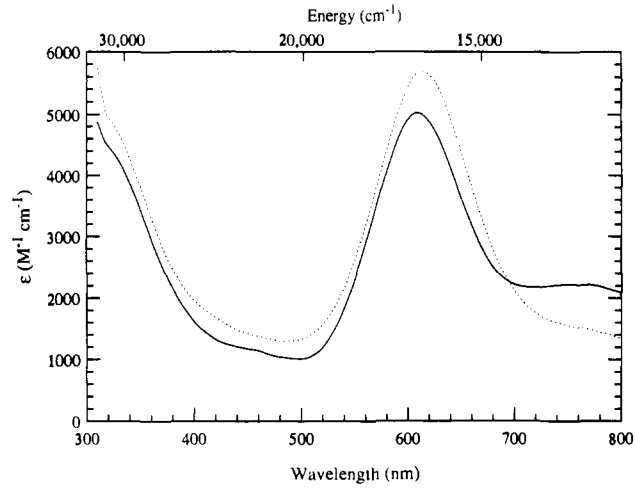

Figure 1. Absorption spectra of ascorbate oxidase and laccase at 277 K: (—) ascorbate oxidase; (···) laccase.

UV–visible absorption spectra were recorded at 277 K on either a Cary 17 or a Hewlett Packard HP8452A diode array spectrophotometer in 2-mm quartz cuvettes. Low-temperature absorption and MCD data were obtained using a sample cell consisting of two quartz disks with a 1.5-mm rubber spacer. Samples were mounted on a laboratory-built optical dewar for 77 K absorption measurements. Low-temperature MCD spectroscopy was performed using a Jasco J 500-C spectropolarimeter operating with S-1 and S-20 photomultiplier tubes for the 1100–800- and 800–300-nm regions, respectively, and an Oxford Instruments Spectromag 4 6-T superconducting magnet/cryostat as previously described.[3,8] MCD intensity is reported in units of $(M\ cm\ T)^{-1}$. EPR spectra were obtained with a Bruker ER 220-D-SRC spectrometer. Sample temperatures of 100–77 K were maintained using a liquid-N_2 finger dewar or a N_2-gas-flow system, and temperatures from 70 to 4 K were obtained using an Air Products Model LTR Helitran liquid-helium transfer refrigerator and a Lake Shore Cryotronics temperature controller, Model DTC-500. The electronic and EPR spectra were smoothed for presentation using a Fourier filtering algorithm.[17]

Results and Analysis

Native Ascorbate Oxidase. Figure 1 shows the absorption spectra of ascorbate oxidase and laccase. Both systems exhibit intense absorption features (centered at 610 nm in ascorbate oxidase and at 614 nm in laccase). By analogy to our assignments of the plastocyanin spectrum,[4] these bands are assigned as type 1 cys $\pi \rightarrow Cu^{2+}$ $d_{x^2-y^2}$ CT. In addition, both enzymes exhibit a 330-nm absorption shoulder that is associated with the type 3 site. The intensity of the 330-nm band in ascorbate oxidase is not affected by addition of up to 30 equiv of H_2O_2.[18] Thus, all of the type 3 sites are oxidized in the native enzyme. In contrast, 22% of the type 3 sites are reduced in native laccase at this pH.[6c]

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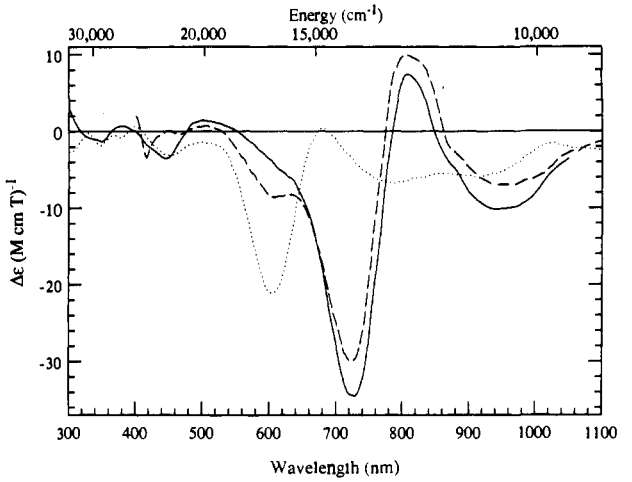

Figure 2. MCD spectra: (—) ascorbate oxidase; (···) fully oxidized laccase; (- - -) plastocyanin. Laccase was treated with 30 equiv of H_2O_2 to oxidize the 22% reduced type 3 sites.[6b] The spectra were recorded under the following conditions: ascorbate oxidase, 4.2 K and 5 T; laccase, 4.8 K and 5 T; plastocyanin, 4.5 K and 4 T. These different conditions will affect the observed amplitudes by less than 10%. The spectrum of laccase is from ref 8b, and the spectrum of plastocyanin is from ref 4.

A weaker band near 750 nm is observed in the spectrum of ascorbate oxidase, but it is not as prominent in laccase. Recent work on plastocyanin indicates that most of the intensity in this region is associated with the + component of the $d_{xz,yz} \rightarrow d_{x^2-y^2}$ ligand-field transition of the rhombically distorted C_{3v} type 1 Cu^{2+} site.[4]

Figure 2 shows the low-temperature MCD spectra of ascorbate oxidase, laccase (treated with 30 equiv of H_2O_2 to oxidize the 22% reduced type 3 sites), and plastocyanin. At 4 K, the MCD spectra of the multicopper oxidases is dominated by paramagnetic *C*-term intensity associated with the type 2 and type 1 sites. In laccase, it has been previously observed that the type 1 site makes a stronger contribution to the MCD spectrum than the type 2 site.[8] First, we consider the ligand-field region (>700 nm). In the MCD spectrum of ascorbate oxidase, intense features are present at (-)730, (+)810, and (-)910 nm. These features correspond closely in both intensity and energy with three d → d features in the plastocyanin MCD spectrum, and thus they are assigned to the type 1 site. Several of these transitions in ascorbate oxidase have previously been observed in room-temperature CD and MCD.[19] The 730- and 810-nm features can now be assigned as the - and + components of the $d_{xz,yz} \rightarrow d_{x^2-y^2}$ transition, and the 910-nm band is assigned as the $d_{xy} \rightarrow d_{x^2-y^2}$ transition. Alternatively, d → d transitions of the laccase type 1 site in the 700–1000-nm region are substantially weaker than those of ascorbate oxidase and plastocyanin, and the energies and signs of the laccase d → d transitions do not directly correspond with the three d → d bands observed in the other type 1 sites. The intensities of *C*-term MCD features are expected to scale with the absorption intensities.[15] In the room-temperature absorption spectra in Figure 1, the 750-nm band in laccase is weaker than that in ascorbate oxidase, and in low-temperature absorption measurements, the intensity near 750 nm in laccase[20] is about 80% that of plastocyanin,[4] whereas in ascorbate oxidase[18] it is about twice that of plastocyanin. Thus, the decreased MCD intensity in the ligand-field region in laccase relative to the other type 1 sites appears to be associated with weaker absorption intensity.

In laccase, an intense negative MCD feature is present at 614 nm, which corresponds in energy with the intense blue copper absorption feature in Figure 1. A similar feature is also present in the plastocyanin MCD spectrum, but it is only 40% as intense, and in ascorbate oxidase, it is <15% as intense as in laccase. From

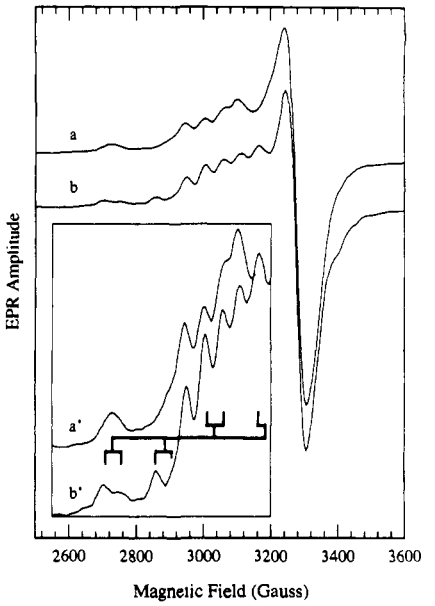

Figure 3. 77 K EPR spectra of resting and fluoride-treated ascorbate oxidase: (a) resting enzyme; (b) resting enzyme + 10 mM F^-. Inset, $g_\parallel$ region at a 3.9 times higher gain; (a') resting enzyme; (b') resting enzyme + 10 mM F^-. The diagram indicates the type 2 Cu^{2+} ^{19}F doublet superhyperfine coupling in the $g_\parallel$ region ($A_F = 57 \times 10^{-4}$ cm^{-1}). Conditions: microwave frequency, 9.439 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 12.5 G; time constant, 0.5 s; sweep time, 500 s.

our previous studies of plastocyanin, this cys $\pi \rightarrow Cu^{2+}$ transition is calculated to have one of the lowest C/D ratios,[4] and thus it is expected to contribute little intensity to the low-temperature MCD spectrum. As predicted, the experimental MCD intensity in this region is indeed quite low for both plastocyanin and ascorbate oxidase. The C/D ratio for plastocyanin has been measured to be -0.009;[4] in ascorbate oxidase, only a weak unresolved shoulder is present at this wavelength, indicating that $|C/D| < 0.01$. In contrast, in laccase, the 614-nm MCD band is the most intense feature in the entire spectrum. In laccase, the 614-nm absorption band sharpens considerably upon cooling[8a,20] such that ϵ_{614} increases from 5700 M^{-1} cm^{-1} at room temperature to ~8000 M^{-1} cm^{-1} at 77 K. In contrast, over the same temperature range, the intensity in ascorbate oxidase increases from 5000 to ~5700 M^{-1} cm^{-1} (data not shown), and in plastocyanin, it increases from 4400 to 5300 M^{-1} cm^{-1}.[21] Thus, at low temperature the C/D ratio for this transition in laccase is ~-0.019, which is ~2-fold larger than for ascorbate oxidase and plastocyanin but significantly less than is observed for the type 1 d → d transitions in ascorbate oxidase and plastocyanin ($|C/D| \sim 0.08$–0.4).[22] Thus, the prominence of the 614-nm MCD feature in laccase derives from its large low-temperature absorption intensity, and it appears that the unusual MCD spectral features in laccase relative to the type 1 sites of ascorbate oxidase and plastocyanin principally reflect a shift in the absorption intensity from the d → d transitions to the thiolate CT transition.

Weaker features are observed in the MCD spectrum of ascorbate oxidase at (-)350, (+)380, and (-)445 nm that are assigned as ligand → Cu^{2+} CT transitions. The band at 445 nm corresponds closely to a feature at the same wavelength in the laccase spectrum[8] and to a band at 465 nm in the plastocyanin spectrum that have been assigned as type 1 imidazole $\pi_1 \rightarrow Cu^{2+}$ CT.[4] The other CT bands are assigned to the type 2 site on the

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(22) The low-temperature MCD spectrum of the type 1 site in *Pseudomanus aeruginosa* azurin exhibits a negative feature at ~630 nm with about half the intensity of the laccase 614-nm MCD; the C/D ratio for this transition is ~-0.020, which is close to that observed for laccase: Greenwood, C.; Hill, B. C.; Barber, D.; Eglinton, D. G.; Thompson, A. J. *Biochem. J.* **1983**, *215*, 303–316.

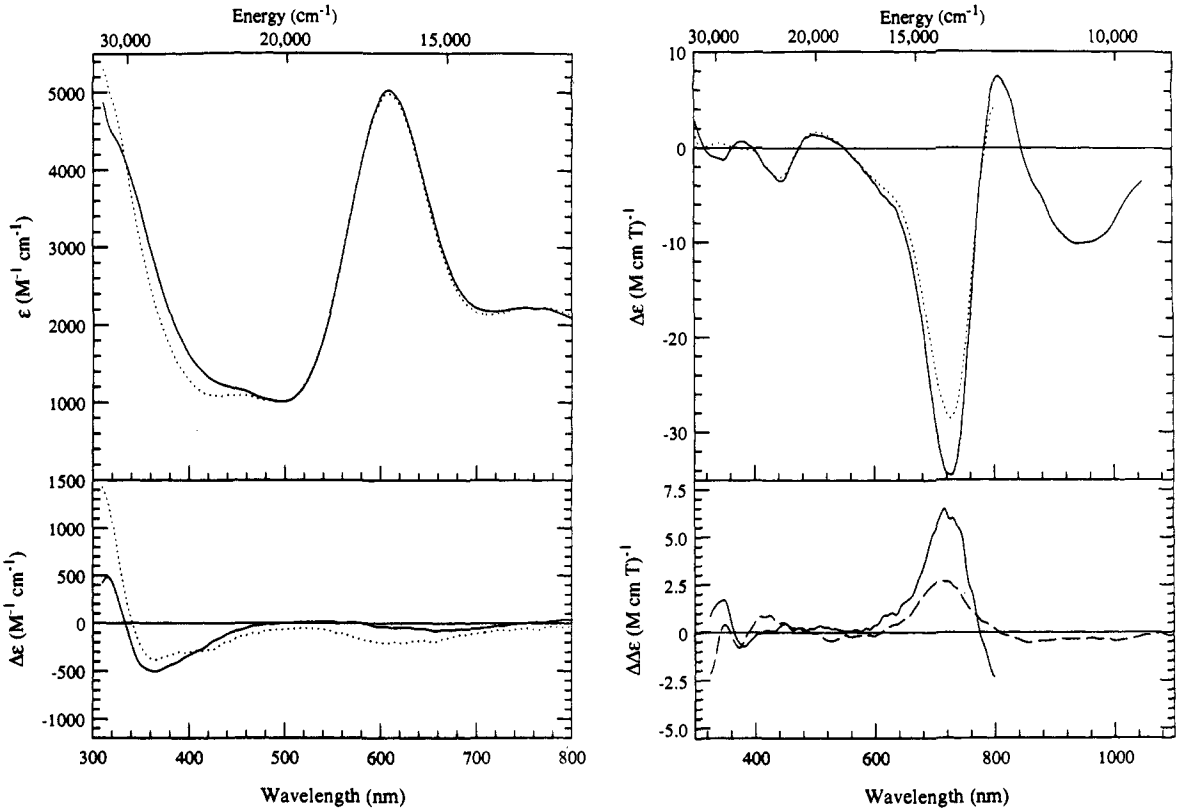

Figure 4. Effects of fluoride binding on the electronic spectra of ascorbate oxidase. (A, Left) Absorption at 277 K: top, spectra for (—) resting enzyme and (···) 10 mM F^-; bottom, absorption difference spectra relative to resting enzyme for (—) ascorbate oxidase + 10 mM F^- and (···) laccase + 10 mM F^-. (B, Right) MCD at 4.2 K and 5 T: top, MCD for (—) resting enzyme and (···) 10 mM F^-; bottom, MCD difference spectra relative to resting enzyme for (—) ascorbate oxidase + 10 mM F^- and (- - -) laccase + 10 mM F^-.

basis of fluoride perturbation effects (vide infra).

Fluoride Binding. We have probed the interaction of fluoride with the trinuclear site in ascorbate oxidase. Figure 3 shows the EPR spectra at 77 K of a control and a sample treated with a large excess of fluoride (10 mM) in a buffer containing 50% glycerol/200 mM potassium phosphate, pH 6. In the fluoride-treated sample, the well-resolved ^{19}F doublet superhyperfine splitting in the $g_{\parallel}$ region ($A_F = 57 \times 10^{-4}$ cm^{-1}) shows that a single fluoride binds equatorially to the type 2 Cu^{2+}. Despite the dominant excited-state spectral contributions from the type 1 site, the type 2 features may be extracted from the absorption and MCD spectra of ascorbate oxidase by their perturbation induced by fluoride binding. Figure 4A shows the effects of fluoride binding on the absorption spectrum of ascorbate oxidase. The 330-nm band becomes sharper, and a slight decrease in intensity is observed in the region between 600 and 700 nm. These changes are clearer in the difference spectrum (Figure 4A, bottom) in which the spectrum of the resting enzyme has been subtracted from the fluoride-treated sample. The difference spectrum shows an increase in intensity at ~310 nm and a decrease at 360 nm, with $\Delta\epsilon = 500$ M^{-1} cm^{-1} at both wavelengths, and a very weak negative band at 680 nm. Also shown in Figure 4A (bottom) is an analogous fluoride difference absorption spectrum of T1Hg laccase.[23] The spectra are generally very similar; in laccase, the 310-nm increase is ~3 times more intense, and the near-IR decrease is centered at 620 nm and is also more intense. Kinetic measurements of the UV absorption changes indicate that fluoride binds with $t_{1/2} \sim 10$ min (10 mM F^-, 25 °C). Figure 4B shows the effects of F^- on the low-temperature MCD spectrum of ascorbate oxidase. Upon fluoride binding at the type 2 site (from EPR superhyperfine couplings), the negative band at 730 nm decreases slightly in amplitude and the CT features at 350 and 380 nm are eliminated. The type 1 His CT band at 445 nm (vide supra) is largely unaffected. These changes are most apparent in the fluoride minus resting difference spectrum (Figure 4B, bottom). Also included in Figure 4B is an analogous fluoride perturbation difference MCD spectrum of T1Hg laccase. In laccase, a fluoride-induced positive MCD change is observed at 720 nm with about half the amplitude of the change in ascorbate oxidase. Laccase also exhibits a similar decrease in the intensity of CT features centered at 345 and 370 nm.

The spectral features associated with fluoride binding in ascorbate oxidase and laccase are extremely similar. In aqueous buffers, two fluorides coordinate to the laccase type 2 Cu^{2+},[25] but in the presence of 50% glycerol, only a single fluoride binds.[8] It has previously been shown that two fluorides also coordinate to the type 2 site in ascorbate oxidase in the absence of glycerol.[26] Figure 3 shows that the presence of 50% glycerol prevents the binding of the second fluoride in ascorbate oxidase, and the magnitude of the fluoride superhyperfine couplings is essentially the same in the monofluoride complex as in laccase (laccase, $A_F = 54 \times 10^{-4}$ cm^{-1}; ascorbate oxidase, $A_F = 57 \times 10^{-4}$ cm^{-1}). In addition, the fluoride-induced electronic spectral perturbations are similar. In both systems, the 330-nm absorption band exhibits a characteristic sharpening upon fluoride binding. In studies of T1Hg laccase, where the spectral contributions from the type 1 center are eliminated, we have demonstrated that both the type 2 and type 3 centers are perturbed by fluoride binding,[3] so it is not possible to definitively assign the absorption change near 330 nm to either center. In T1Hg laccase, the type 2 electronic features are clearly resolved in the MCD spectrum; fluoride binding

(23) The laccase type 1 Cu^{2+} may be selectively replaced by Hg^{2+}, leaving the type 2 and type 3 sites intact;[10,24] this form is referred to as type 1 Hg^{2+}-substituted (T1Hg) laccase. Fluoride binds similarly in native and T1Hg laccase.[3,24b]

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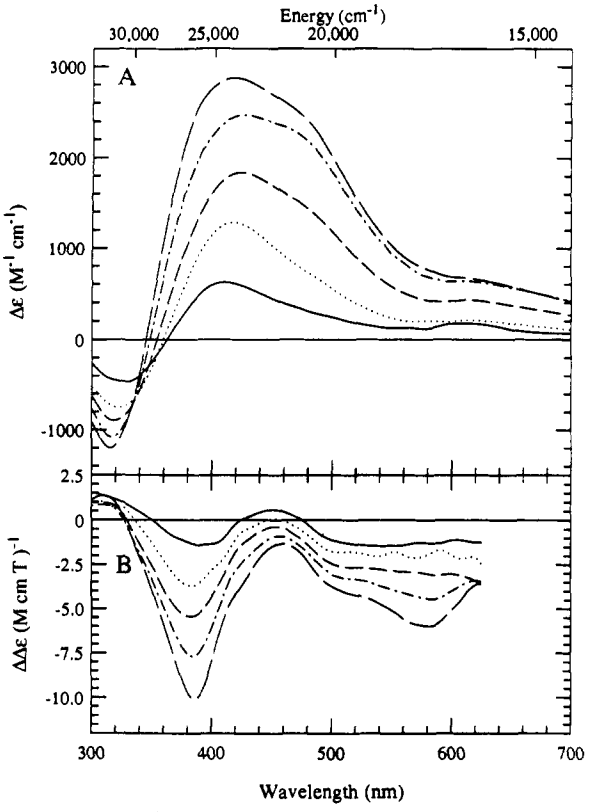

Figure 5. Titration of ascorbate oxidase with N_3^- probed by charge-transfer difference spectra relative to resting enzyme: (A) absorption at 277 K; (B) MCD at 4.2 K and 5 T. N_3^- concentration: (—) 0.5 mM; (···) 2 mM; (- - -) 5 mM; (-·-) 20 mM; (— — —) 50 mM.

dramatically decreases the amplitudes of both the d → d bands at 860 and 720 nm and the CT features at 320, 345, and 370 nm. In addition, a decrease in absorption intensity near 600 nm is associated with a perturbation of the type 2 $d_{xz,yz} \rightarrow d_{x^2-y^2}$ transition.[3] Very similar perturbations are observed in ascorbate oxidase (Figure 4), and these changes are therefore assigned to the type 2 site. Thus, the features in the ascorbate oxidase MCD spectrum at 350 and 380 nm are assigned as type 2 imidazole → Cu^{2+} CT transitions. It is also clear that the 730-nm type 1 MCD feature contains a contribution from a type 2 d → d transition. These data indicate that the electronic and geometric structures of the type 2 sites are similar in the two enzymes, i.e. five- or six-coordinate tetragonal Cu^{2+}. In addition, the fluoride EPR superhyperfine splittings in Figure 3 indicate similar equatorial fluoride bindings to the type 2 Cu^{2+} centers in ascorbate oxidase and laccase.

Azide Binding. Figure 5 shows the absorption and low-temperature MCD difference spectra produced upon titrating ascorbate oxidase with 0.5–50 mM azide. In the absorption spectrum, an intense broad feature is observed near 410 nm with a shoulder at ~485 nm. On the basis of a comparison with laccase and Cu^{2+} azide model complexes, these features are assigned as $N_3^- \rightarrow Cu^{2+}$ CT bands. An absorption decrease is observed in the UV region, which is associated with perturbations of the 330-nm band of the type 3 site. In native laccase, the 22% of the trinuclear sites which contain reduced type 3 sites give rise to "high-affinity" azide binding with $K > 10\,000$ M^{-1}.[9c,8,27] The spectral features associated with high-affinity azide binding include $N_3^- \rightarrow$ type 2 Cu^{2+} CT absorption intensity extending from 410 to 500 nm ($\Delta\epsilon \sim 500$ M^{-1}) and $N_3^- \rightarrow$ type 2 Cu^{2+} MCD bands at (+)445 and (−)510 nm.[8] Although a CT band is observed in ascorbate oxidase near 500 nm (Figure 5), this feature has a much lower binding constant than the high-affinity azide in laccase (vide infra). The extinction coefficient for the 485-nm absorption feature in ascorbate oxidase is ~2300 M^{-1} cm^{-1}. Typically, $N_3^- \rightarrow Cu^{2+}$ CT bands have extinction coefficients of ~2000 M^{-1} cm^{-1}, indicating that the 485-nm band cannot arise from a minority fraction of sites that are reduced. Thus, the azide spectral features in ascorbate oxidase are associated with fully oxidized sites. This interpretation is consistent with the observation that no reduced-type centers are present in our ascorbate oxidase preparation (vide supra). Two previous studies reported high-affinity azide binding to ascorbate oxidase[13a,d] whereas others have not observed this effect.[13b,c,e] It is noteworthy that high-affinity azide binding is not observed in single crystals of the enzyme.[13e]

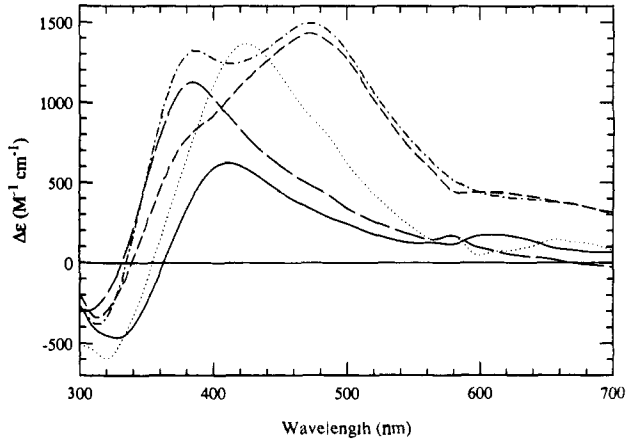

Figure 6. Charge-transfer absorption spectra of N_3^- complexes of ascorbate oxidase calculated from differences in the spectra given in Figure 5A. N_3^- concentration: (—) 0.5–0 mM; (···) 2–0.5 mM; (- - -) 5–2 mM; (-·-) 20–50 mM; (— — —) 50–20 mM.

In the MCD titration, the main spectral features are a strong negative band at 385 nm, a weak positive feature near 460 nm, a negative shoulder near 500 nm, and a negative band at 590 nm (Figure 5B). Azide CT absorption spectra were also recorded at 277 and 77 K for $[N_3^-] = 5$ and 50 mM; although the resolution of the 410- and 485-nm bands is increased at low temperature, their positions do not change with temperature (data not shown). Thus, the 385-nm MCD feature (Figure 5B) is associated not with the main absorption band at 410 nm (Figure 5A) but instead with another absorption band centered near 385 nm. This absorption feature is not observed in the titration of Figure 5A, owing to overlap with the strong band at 410 nm, but it is clearly resolved in the replotted data shown in Figure 6 (vide infra). Because this 385-nm band gives rise to *C*-term MCD intensity, it is assigned as $N_3^- \rightarrow$ type 2 Cu^{2+} CT. In contrast, the 410-nm absorption band does not give rise to *C*-term MCD intensity, and it is assigned as $N_3^- \rightarrow$ coupled type 3 Cu^{2+} CT. Because several MCD features are observed near 500 nm, assignment of the 485-nm absorption band to the type 2 or the type 3 site is difficult using only the azide titration data; however, kinetic measurements indicate that it is associated with the type 3 site (vide infra).

In Figure 6, the absorption titration data of Figure 5A have been replotted to determine the relative binding constants of the three CT bands; each spectrum is the difference between a given azide concentration and the next lower concentration. The only feature present in the 0.5–0 and the 2–0.5 mM spectra is the 410-nm band. The 5–2 mM spectrum contains a dominant contribution from the 485-nm band, a weak shoulder at 385 nm. The relative intensity of the 385-nm band increases in the 20–5 mM spectrum. Finally, the 50–20 mM spectrum only contains the 385-nm band. Thus, the three CT features have different binding constants with $K_{410} > K_{485} > K_{385}$, indicating that three different azides bind to the ascorbate oxidase trinuclear site. The absolute binding constants are difficult to extract from the absorption data owing to the strong overlap of the three CT bands. Analysis of Gaussian-resolved data indicates that the binding constant at 410 nm is ~1000 M^{-1} and the binding constant at 485 nm is ~200 M^{-1}. The binding constant of the 385-nm band cannot be readily obtained owing to the overlap with the 330-nm perturbation; but clearly $K_{385} \ll 200$ M^{-1}.

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Table I. Comparison of Azide Bindings in Laccase and Ascorbate Oxidase[a]

parameter	laccase		ascorbate oxidase		
	first azide	second azide	azide A	azide B	azide C
K, M^{-1} [b]	780	270	~1000	~200	≪200
k_{on}, min^{-1}	>1	>1	>1	0.003	>1
$N_3^- \rightarrow$ type 2 Cu^{2+} CT[c]	500 (500)				385 (>2000)
$N_3^- \rightarrow$ type 3 Cu^{2+} CT[c]	340 (1000)	400 (1900)	410 (1900)	485 (2300)	
type 2 d → d MCD	unchanged	shift + 1400 cm^{-1}	shift + 1500 cm^{-1}	unchanged	unchanged
type 2 EPR $A_\parallel$, cm^{-1} [d]	unchanged	140 × 10^{-4}	160 × 10^{-4}	unchanged	unchanged

[a] Laccase data are taken from ref 3. [b] Binding constants measured at 4 °C. [c] CT wavelength in nm (molar extinction coefficient in parentheses). [d] In unligated laccase, the type 2 $A_\parallel$ = 200 × 10^{-4} cm^{-1},[23b] and in ascorbate oxidase, type 2 $A_\parallel$ = 190 × 10^{-4} cm^{-1}.[12a]

The rate constants for the formation of the $N_3^- \rightarrow Cu^{2+}$ CT bands are quite different. Figure 7 shows the kinetics of azide binding as monitored by absorption and low-temperature MCD spectroscopy. In the presence of 50 mM azide, the 385- and 410-nm bands are maximized within 1 min, indicating that $k >$ 1 min^{-1}. In contrast, the 485-nm shoulder develops slowly over the course of 24 h. This feature is most well resolved in the difference absorption spectra (Figure 7B), in which the 30-min spectrum has been subtracted. The rate of increase in the 485-nm band is independent of $[N_3^-]$ between 5 and 50 mM with a first-order rate constant of k = 0.003 min^{-1} ($t_{1/2}$ ~ 4 h). In separate experiments, the azide dissociation rates were studied by equilibrating samples at 50 mM azide for 24 h and then rapidly removing the excess azide either by dilution or by gel filtration chromatography. It was observed that the 385- and 410-nm bands disappear immediately upon removing the ambient azide, but the 485-nm band remains and subsequently decays over the course of several hours. Thus, the CT features at 385 and 410 nm are independent of the 485-nm band, thus confirming that a different azide gives rise to the 485-nm CT band.

Figure 7C shows the MCD difference spectra taken 45 min and 24 h after addition of 50 mM azide. A strong negative 385-nm band is observed in the 45-nm spectrum, and this feature increases by about 30% after 24 h. In addition, a weaker band near 590 nm with a shoulder near 500 nm is present in the 45-min spectrum; these features increase by 40–50% over 24 h. The kinetics of the 385-nm band are consistent with the absorption data, indicating that this azide binds rapidly. There is no intense MCD feature that parallels the kinetics of the 485-nm absorption band. The extinction coefficients of the 485- and 385-nm absorption bands are about equal, but the increase in the MCD intensity at 485 nm between the 45-min and 24-h spectra only amounts to about 10–20% of the intensity of the 385-nm band. The lack of a strong MCD feature associated with the 485-nm absorption band suggests that it should be assigned to an azide bound to the type 3 site. The 500–590-nm MCD intensity likely reflects a perturbation of the paramagnetic type 1 site induced by azide binding at the type 2 and type 3 centers.

In summary, the $N_3^- \rightarrow Cu^{2+}$ CT features at 385, 410, and 485 nm exhibit differences in both equilibrium binding constants and binding kinetics, indicating that three different azides coordinate to the trinuclear site in ascorbate oxidase. For convenience, the three azide molecules are labeled on the basis of their absorption maxima: azide A, 410 nm; azide B, 485 nm; azide C, 385 nm. The thermodynamic, kinetic, and spectroscopic properties of these azides are summarized in Table I. Note that the differences in both the equilibrium binding constants and the binding kinetics of the three azides provide a useful methodology for assigning spectroscopic features in absorbance, low-temperature MCD, and EPR (vide infra) measurements to a given azide molecule.

Figure 8 presents the effects of azide binding on the low-temperature MCD spectrum of ascorbate oxidase in the ligand-field region. In the equilibrium azide titration (Figure 8A), the dominant spectral features in the azide minus resting difference spectra are a negative band at 690 nm, a positive band at 770 nm, and weaker features at (−)845 and (+)1010 nm. In principle, both the type 1 and type 2 centers can exhibit C-term MCD and these features could be associated with either the type 1 or the type 2 d → d transitions. The positive band at 770 nm occurs at approximately the same wavelength as the negative type 2 band at

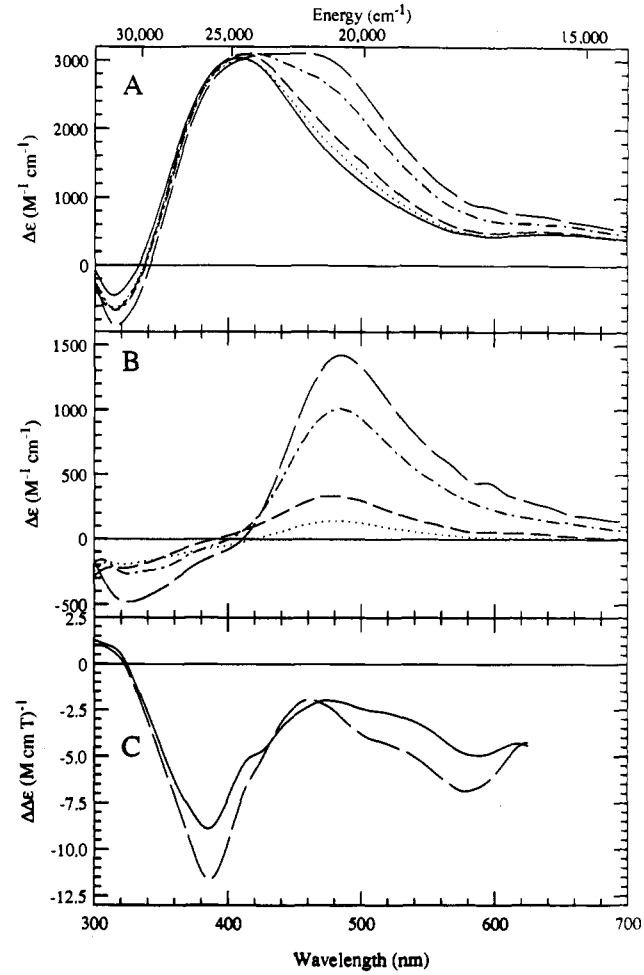

Figure 7. Kinetics of N_3^- binding ($[N_3^-]$ = 50 mM, temperature 277 K). (A) absorption difference spectra relative to resting enzyme and (B) absorption difference spectra relative to 30-min sample: (—) 0.5 h; (···) 1 h; (- - -) 2 h; (-·-) 8 h; (— — —) 24 h. (C) MCD (4.2 K and 5 T) difference spectra relative to resting enzyme: (—) 45 min; (— — —) 24 h.

730 nm (see Figure 4B, bottom), and the changes at 770 and 690 nm are consistent with a shift in position of the type 2 feature by 1500 cm^{-1} to higher energy. Indeed, binding of the second azide to T1Hg laccase causes a very similar perturbation of the type 2 feature, causing it to shift 1400 cm^{-1} to higher energy.[3] Thus, the changes at 770 and 690 nm are assigned to a perturbation of the electronic structure of the type 2 site. We have also characterized the kinetics of the ligand-field perturbations (Figure 8B). The 690- and 770-nm features in the difference spectra are maximized within 45 min of exposure to azide, indicating that the type 2 d → d perturbation is associated with the rapidly binding azides: A or C. The binding constant for the ligand-field perturbation is ~750 M^{-1} (vide supra), which is close to the value for azide A determined from the CT absorption features (1000 M^{-1}, Table I), but it is much greater than the binding constant for azide C (≪200 M^{-1}). The features at 845 and 1010 nm in the difference spectrum (Figure 8A) are caused by a ~30%

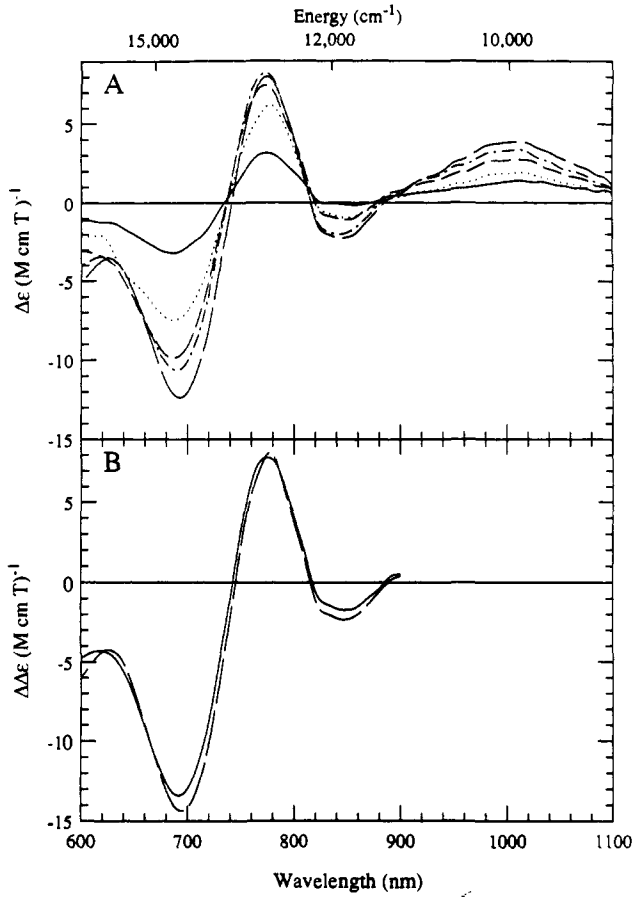

Figure 8. MCD spectra of N_3^--bound ascorbate oxidase at 4.2 K and 5 T in the ligand-field region: (A) N_3^- titration for (—) 0.5 mM N_3^-, (···) 2 mM N_3^-, (- - -) 5 mM N_3^-, (-·-) 20 mM N_3^-, and (— — —) 50 mM N_3^-; (B) kinetics of N_3^- binding for (—) 45 min and (— — —) 24 h. Difference spectra are relative to resting enzyme.

decrease in the amplitude of the (+)810- and (−)940-nm type 1 d → d MCD transitions present in the resting enzyme (Figure 2). In Figure 8B, the type 1 perturbation at 845 nm is maximized in the 45-min spectrum, indicating that azide A or C perturbs the type 1 site in ascorbate oxidase.

Azide binding also produces characteristic changes in the EPR spectrum of the type 2 site. Figure 9 shows the 77 K EPR spectra for a series of ascorbate oxidase samples equilibrated with 0.5–50 mM N_3^-. In the $g_{\|}$ region, the low-field $M_I = -3/2$ hyperfine line of the type 2 Cu^{2+} shifts to higher field (see inset), and by a concentration of 20 mM azide, the spectrum is converted to the azide-perturbed form characterized by decrease in $A_{\|}$ from 190 × 10^{-4} to ~160 × 10^{-4} cm^{-1}. The concentration dependence of the type 2 perturbation indicates that $K > 500$ M^{-1}. Figure 10 shows the kinetics of the 77 K EPR changes induced by addition of 50 mM azide. It is apparent that the type 2 perturbation is complete within 45 min, and no additional change in the EPR spectrum occurs over the course of 24 h. Thus, the >500 M^{-1} binding constant and the rapid kinetics indicate that the perturbation of the type 2 EPR spectrum is assigned to binding of azide A.

In laccase, two different azide molecules bind to the fully oxidized trinuclear site with different equilibrium constants. Binding of the "first azide" (K_1 = 780 M^{-1}) results in the displacement of the endogenous bridge in a limited fraction (<10%) of the type 3 sites, thereby disrupting the antiferromagnetic coupling between the coppers and rendering these sites paramagnetic.[3,8] These uncoupled sites give rise to $N_3^- \rightarrow Cu^{2+}$ CT MCD features at 340, 385, and 440 nm and to MCD d → d features. In addition, a characteristic zero-field-split triplet EPR spectrum is observable at liquid-helium temperature. These features decrease in amplitude upon binding of the "second azide"

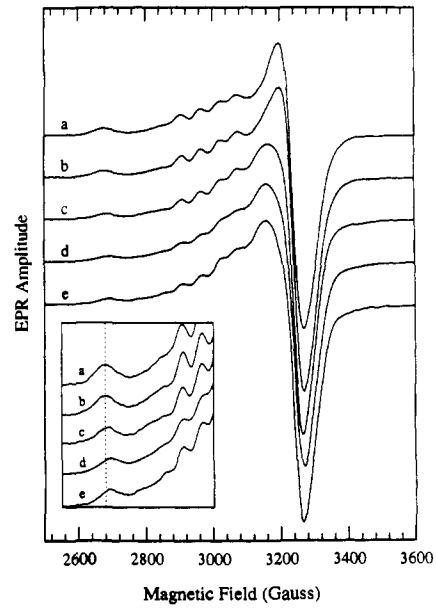

Figure 9. Titration of ascorbate oxidase with N_3^- probed by 77 K EPR spectra: (a) resting enzyme; (b) 0.5 mM N_3^-; (c) 5 mM N_3^-; (d) 20 mM N_3^-; (e) 50 mM N_3^-. Conditions: microwave frequency, 9.315 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 12.5 G; time constant, 0.2 s; sweep time, 200 s.

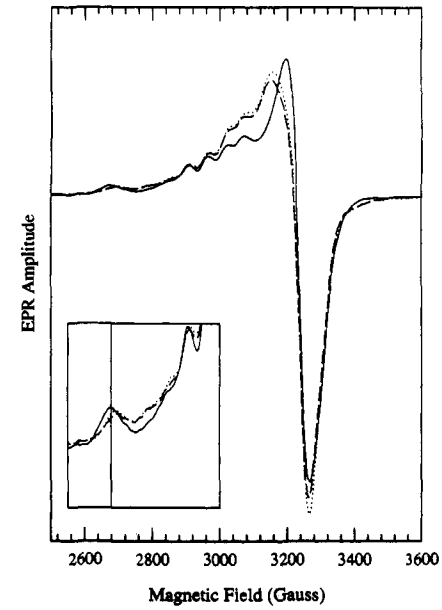

Figure 10. Kinetics of N_3^- binding illustrated by 77 K EPR spectra: (—) resting enzyme; (- - -) sample frozen 45 min after addition of 50 mM N_3^-; (···) sample frozen 24 h after addition of 50 mM N_3^-.

(K_2 = 270 M^{-1}), indicating that the second azide recouples the <10% uncoupled type 3 sites. In contrast to laccase, we observe no $N_3^- \rightarrow Cu^{2+}$ CT features in the MCD spectrum that are associated with uncoupled type 3 sites in the ascorbate oxidase azide titrations and in the kinetic studies. In addition, no dipolar coupled triplet signals are observed in the EPR spectra of these samples recorded at 5 K.

Discussion

The application of absorption, MCD, and EPR spectroscopies to the study of ascorbate oxidase has provided new insights into the geometric and electronic structures of both the type 1 and the type 2–type 3 trinuclear copper cluster in this enzyme. It is generally thought that the type 1 sites in the multicopper oxidases are closely related to type 1 copper protein plastocyanin, which contains a Cu^{2+} center ligated by two histidines, a cysteine, and an axial methionine weakly bound at ~2.9 in a distorted C_{3v}

geometry.[4,28] Indeed, in the ascorbate oxidase crystal structure,[5] the type 1 Cu^{2+} domains are nearly superimposable upon those of the plastocyanin structure.[28] The other multicopper oxidases have not been crystallographically defined, but considerable spectroscopic data are available. The laccase type 1 ligand environment has been examined by comparison of the Hg EXAFS and XANES of T1Hg laccase with those of the Hg^{2+}-substituted type 1 proteins, and it was concluded that the laccase type 1 ligation is similar to those of plastocyanin and azurin.[29] The energies of the absorption and CD transitions of laccase,[19] ascorbate oxidase,[18] and plastocyanin[4] are quite close. Similar structural features in the type 1 Cu^{2+} sites in ascorbate oxidase, laccase, and plastocyanin have been inferred from the similar Raman vibrational frequencies observed in these systems.[30] Although the EPR parameters of the type 1 sites in ascorbate oxidase and plastocyanin are quite similar, in laccase $A_{\parallel}$ is significantly less (laccase, 43×10^{-4} cm^{-1}; ascorbate oxidase, 58×10^{-4} cm^{-1}; plastocyanin, 63×10^{-4} cm^{-1}).[12] ENDOR measurements indicate that the histidine ^{14}N couplings in tree laccase are unique among the blue copper sites that were studied.[31]

In the present study, it is observed that the low-temperature MCD spectrum of the ascorbate oxidase type 1 site is very similar to the plastocyanin spectrum, but in contrast, the MCD spectrum of laccase is different from the spectra of these other type 1 sites. Comparison of the corresponding absorption spectra indicates that the unusual MCD spectrum of laccase primarily reflects a shift in the absorption intensity from the d → d transitions into the thiolate CT transition. In plastocyanin, the absorption band at 780 nm gains intensity due to mixing of the d_{xy+yz} orbital with the thiolate sulfur p_π orbital.[4] In addition, this mixing splits the d_{xz+yz} and d_{xz-yz} orbitals, producing the pseudo-*A*-term[32] MCD at (−)730 and (+)810 nm in plastocyanin and in ascorbate oxidase. A decrease in this mixing would decrease the absorption intensity of the $d_{xz+yz} \rightarrow d_{x^2-y^2}$ transition and increase the intensity of the cys $\pi \rightarrow Cu^{2+}$ transition. In addition, the amplitude of the d → d transitions at 730 and 810 nm in the MCD spectrum would be further decreased owing to increased spectral overlap of these oppositely signed pseudo-*A*-term components. Thus, the absorption and MCD data indicate that the Cu^{2+} d_{xz+yz}–sulfur p_π mixing is decreased in laccase and the structure of the blue copper site in laccase must therefore be perturbed relative to that in plastocyanin and ascorbate oxidase. Kinetic measurements have demonstrated that the type 1 site in the multicopper oxidases serves to shuttle electrons from exogenous reductants to the type 2–type 3 trinuclear site.[33] Changes in the cys S–p_π Cu^{2+} bonding interaction could affect the cys S character of the redox-active orbital and its orientation, which could result in differences in the reactions of these enzymes with exogenous reductants or differences in the intramolecular electron-transfer reactivity.

The electronic spectrum of the type 2 site in ascorbate oxidase has been revealed by fluoride perturbation of the MCD spectrum (Figure 4, bottom). The energies of the $d_{xy} \rightarrow d_{x^2-y^2}$ and imidazole → Cu^{2+} CT transitions are very similar in ascorbate oxidase and laccase. Thus, the sites have similar tetragonal coordination geometries.

A clear difference between ascorbate oxidase and laccase is that azide binding does not uncouple the type 3 endogenous bridge in ascorbate oxidase. In laccase, the endogenous bridge mediates strong antiferromagnetism ($-2J > 500$ cm^{-1}) between the type 3 coppers via a superexchange mechanism,[2,3] and similar strong antiferromagnetism is observed in ascorbate oxidase.[34] In the ascorbate oxidase crystal structure, the endogenous bridge is observed to be a water-derived ligand;[5] on the basis of the strong antiferromagnetism, this ligand is likely hydroxide. In laccase, binding of the first azide results in the uncoupling of a small fraction (<10%) of the type 3 sites.[3,8] This uncoupling increases at low pH and involves protonation and competitive displacement of the endogenous bridge by azide. Similar uncoupling behavior is observed in ligand-binding studies of met hemocyanin.[35] In contrast, no uncoupled spectral features have been observed in ascorbate oxidase azide titrations or in the studies of azide association and dissociation kinetics down to pH 5.2. This absence of uncoupling may relate to differences in the chemical environment of the endogenous bridge in the two enzymes. The magnitude of uncoupling is dependent on the binding constant of the endogenous bridge with the type 3 coppers and on the intrinsic pK_a of the endogenous bridge.[35] Given that the endogenous bridge is likely hydroxide in both enzymes, the absence of uncoupling in ascorbate oxidase may indicate that either the binding constant of the bridge is increased or the bridge pK_a is lowered in ascorbate oxidase due to differences in the dielectric constant of the type 3 environment or a specific electrostatic interaction with a nearby cationic residue. Alternatively, the absence of uncoupling in ascorbate oxidase may relate to the differences in the mode of azide coordination to type 3 copper (vide infra).

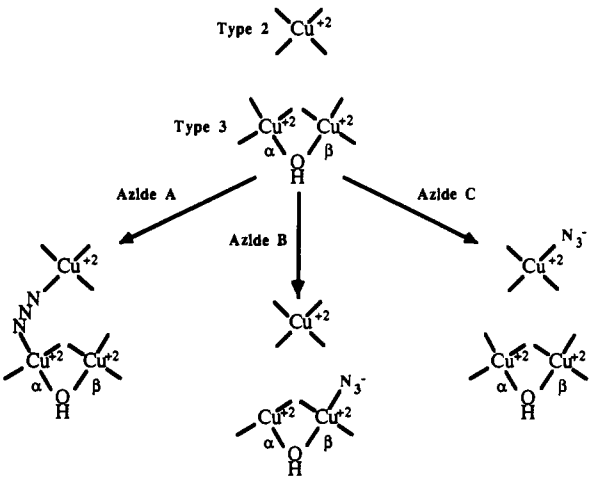

Figure 11. Spectroscopic model for azide binding to the ascorbate oxidase trinuclear copper site. Note that binding of azides A, B, and C is not mutually exclusive and under appropriate conditions all three azides may coordinate to the trinuclear site.

The reactivity of the ascorbate oxidase trinuclear copper center with exogenous ligands reveals interesting similarities and differences relative to the exogenous ligand coordination chemistry of the laccase site. The type 2 EPR, absorption, and MCD features are similarly perturbed upon F^- binding in ascorbate oxidase and laccase. In addition, the presence of 50% glycerol blocks the binding of a second fluoride. These observations suggest that fluoride binds in similar manners in the two systems. In laccase, the type 3 ligand-field bands are perturbed in parallel with the type 2 spectral features, suggesting that fluoride bridges the type 2 and the type 3 sites.[3] This strong interaction of fluoride with the type 3 site provides an explanation for the unusually high binding constant for laccase (K is $>10^4$ times higher than that for aqueous Cu^{2+}).[22b] The F^--binding constant for ascorbate oxidase is ~1000 times higher than that for aqueous Cu^{2+}, suggesting that F^- also interacts with the oxidized type 3 site in this system. In summary, the spectroscopic and thermodynamic similarities of fluoride binding in laccase and ascorbate oxidase

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strongly suggest that fluoride bridges the type 2 and type 3 centers in ascorbate oxidase. Crystallography has suggested the possibility that a μ_3-bridging water-derived ligand may lie in the middle of the triangle formed by the type 2 and type 3 coppers.[5] Thus, it is possible that fluoride coordinates in a μ_3 mode by replacing this water-derived ligand.

Analysis of the thermodynamics and kinetics of azide binding to ascorbate oxidase indicates that three different azide molecules, labeled A, B, and C, coordinate to the trinuclear site (Table I, Figure 11). The spectral features associated with the binding of azide A to ascorbate oxidase are strongly paralleled by the binding of the "second azide" in laccase. These include an $N_3^- \rightarrow Cu^{2+}$ type 3 CT band at 400–410 nm (Figure 5A), a shift in the type 2 d → d band 1400–1500 cm^{-1} to higher energy (Figure 8), and a similar perturbation of the type 2 EPR spectrum (Figure 9). In both systems, these changes are associated with a rapid azide-binding rate. The strong similarity in the spectral changes indicates that azide A binds to ascorbate oxidase in a manner similar to that of the binding of the "second azide" in laccase. The diamagnetic type 3 CT band at 410 nm is observed in absorption but not MCD, which requires that azide A directly coordinate to the type 3 site. The large perturbation of the type 2 electronic structure provides strong evidence that azide A also coordinates to the type 2 Cu^{2+}. Our previous results indicated that the "second azide" bridges the type 2 and type 3 sites in laccase,[3] and a similar binding mode is thus determined for azide A in ascorbate oxidase. In laccase, the type 3 coppers are inequivalent and are labeled α and β, and it was suggested that the first and second azides bind to the β and α coppers, respectively.[3] Thus, it is likely that azide A in ascorbate oxidase binds to only one of the type 3 coppers, which is analogous to the laccase type 3_α. One difference between the two enzymes is that for ascorbate oxidase the binding constant for azide A is ~4 times higher than that for laccase. This difference corresponds to only ~1 kcal/mol difference in $\Delta G°$ of binding, which may reflect small differences in weak interactions of the bound azide with the protein environment.

Binding of azide B to ascorbate oxidase is characterized by a low-energy $N_3^- \rightarrow Cu^{2+}$ CT band at 485 nm that develops with $t_{1/2} \sim 4$ h (Figure 7A,B, Table I). The lack of MCD intensity associated with this transition indicates that it is associated with the type 3 site. The binding constant of azide B is ~200 M^{-1}, which is within the normal range observed for Cu^{2+} azide model complexes and protein systems. However, the kinetics of ligand binding are unusually slow relative to those of Cu^{2+} model complexes ($k = 0.003$ min^{-1}), and the rate is insensitive to $[N_3^-]$ in the range from 5 to 50 mM. For aqueous Cu^{2+} systems with exchangeable axial positions, ligand substitution rates of $>10^8$ M^{-1} s^{-1} are observed.[36] Blocking of the axial position in $[Cu(tren)H_2O]^{2+}$ complexes reduces the on rates by a factor of ~3000.[37] Steric hindrance and solvent accessibility also affect ligand substitution kinetics. Coates et al.[38] have compared the N_3^- anation kinetics of $[Cu(tren)H_2O]^{2+}$ and $Cu[(Me_6tren)H_2O]^{2+}$. Loss of N_3^- from $Cu[(Me_6tren)N_3^-]^+$ occurs ~10^5 times slower than that from $[Cu(tren)N_3^-]^+$. This rate decrease was attributed to, in part, the inaccessibility of the leaving group to solvation within the sterically hindered, hydrophobic environment. These effects may contribute to the slow ligand-binding kinetics in ascorbate oxidase. However, the azide-binding rate for this enzyme is ~10^4 times slower than that for the $Cu[(Me_6tren)N_3^-]^+$ model, suggesting that additional effects are operative (vide infra).

Slow ligand substitution chemistry has previously been observed in studies of other multicopper oxidases. It has been reported that the rate of F^- binding to laccase is independent of $[F^-]$ with $k_{obs} = 9 \times 10^{-4}$ s^{-1} for the fungal enzyme and $k_{obs} = 6 \times 10^{-5}$ s^{-1} for the tree enzyme.[25a] The following mechanism was proposed:

$$\text{laccase} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{laccase}^*$$

$$\text{laccase}^* + F^- \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{laccase–}F^- \qquad (1)$$

with formation of the activated intermediate laccase* representing the rate-limiting step. Proton and ^{17}O NMR relaxation measurements[39] and EPR studies[40] suggest that equatorial water on the type 2 copper in laccase exchanges very slowly with bulk solvent, and it has been proposed that the type 2 and type 3 sites occupy a common cavity which is sequestered from solvent.[39] An activated intermediate model analogous to (1) for the water exchange was proposed, in which a rate-limiting protein conformational change opens the cavity to the solvent. It has been reported that the anation of tree laccase by azide is also slow relative to the anation of $[Cu(tren)H_2O]^{2+}$,[41] and a mechanism was proposed involving formation of an outer-sphere complex followed by a rate-limiting dissociative interchange reaction to give the inner-sphere azide–laccase complex

$$\text{laccase} + X^- \overset{K_{OS}}{\rightleftharpoons} \text{laccase}\cdot X^- \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} \text{laccase–}X^- \qquad (2)$$

($k'_2 = 1.25 \times 10^{-1}$ s^{-1}, $k'_{-2} = 1.5 \times 10^{-2}$ s^{-1}, pH 6.1, phosphate buffer).[41b]

Either mechanism 1 or 2 can account for the slow kinetics of azide B binding to ascorbate oxidase. If mechanism 1 is correct, then formation of ascorbate oxidase* cannot be associated with opening of the pocket containing the type 2 and type 3 sites,[39] because azides A and C bind to the type 2 and type 3 sites with $k > 1$ min^{-1} and the type 2 site in ascorbate oxidase is readily accessible to a variety of small-molecule chelators.[14] Thus, the formation of ascorbate oxidase* would have to represent a specific conformational change that alters the accessibility or the affinity of the type 3 site specifically for azide B. Alternatively, if mechanism 2 is correct, the absence of CT bands prior to the direct binding of azide B to the type 3 site indicates that the outer-sphere complex does not involve direct binding to Cu^{2+}. One possibility is that azide B first binds to an amino acid side chain in the vicinity of the type 3 site. Indeed, the $N_3^- \rightarrow Cu^{2+}$ CT features of the final azide B–type 3 adduct suggest that this azide strongly interacts with a protein side chain.

The single $N_3^- \rightarrow Cu^{2+}$ CT transition at 485 nm observed for azide B is quite unusual. In terminal Cu^{2+} azide complexes, a single absorption band associated with the N_3^- $\pi_\sigma \rightarrow Cu^{2+}$ transition generally occurs at ~400 nm with $\epsilon \sim 2000$ M^{-1} cm^{-1}.[42] The other possible transition, N_3^- $\pi_v \rightarrow Cu^{2+}$, is not usually observed in absorption due to poor overlap with the $d_{x^2-y^2}$ orbital. However, a π_v transition near 500 nm is observed in the absorption spectra of the first azide in laccase ($\epsilon \sim 500$ M^{-1} cm^{-1})[3] (Table I) and in met azide *Limulus* hemocyanin ($\epsilon = 1000$ M^{-1} cm^{-1}),[42] where azide bridges two Cu^{2+} ions in a μ-1,3 geometry. This feature gains intensity through mixing of the π_σ and π_v orbitals induced by distortion of the coordinated azide. This distortion may involve a deviation of the $Cu–N_3^–Cu$ dihedral angle from planarity or a strong interaction with a nearby residue. In both of these systems, more intense CT features are observed near 400 nm, and they are assigned as N_3^- $\pi_\sigma \rightarrow Cu^{2+}$ transitions. In contrast, no higher energy band is observed for azide B in ascorbate oxidase. There are two possible assignments for the 485-nm CT feature of azide B: (1) an unusually low energy $\pi_\sigma \rightarrow Cu^{2+}$ transition in a terminal binding geometry or (2) $\pi_v \rightarrow Cu^{2+}$ in

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a bridged system. Assignment 1 requires that the $\pi_\sigma \rightarrow Cu^{2+}$ transition shift $\sim$4000 cm^{-1} to lower energy relative to that of other Cu^{2+} azide complexes, which is unlikely. Alternatively, if the transition is $\pi_v \rightarrow Cu^{2+}$, then a strong bonding interaction of azide B with another species is required to provide large overlap between with the azide π_v and type 3 Cu^{2+} $d_{x^2-y^2}$ orbital. This species may be the amino acid side chain in the vicinity of the type 3 site that is involved in formation of the kinetically defined outer-sphere azide complex described above. Thus in the context of this model, formation of the 485-nm band may represent an intramolecular isomerization of the bound azide from an outer-sphere azide complex to a bridging geometry between an amino acid side chain and the type 3 site.

Binding of azide C to ascorbate oxidase is characterized by a $N_3^- \rightarrow$ type 2 Cu^{2+} CT band at 385 nm which has MCD intensity and a low binding constant of $\ll$200 M^{-1} (Figure 7A,B, Table I). The absorption band is assigned as an N_3^- $\pi_\sigma \rightarrow$ type 2 Cu^{2+} transition, and the energy and amplitude are typical of terminal Cu^{2+} azide complexes. Consistent with our observations that two azides (A and C) bind to the type 2 Cu^{2+}, two equatorial open coordination positions are observed in the crystal structure of ascorbate oxidase.[5] In this structure, one of these positions faces toward and one position faces away from the type 3 coppers. It is reasonable to associate azide C with the position facing away and azide A with the position facing toward and bridging to the type 3 coppers. The spectroscopically effective model for exogenous ligand interactions with the ascorbate oxidase trinuclear site is summarized in Figure 11.

In laccase, binding of the first azide is characterized by a type 2 CT feature at 500 nm and a type 3 CT feature at 340 nm, indicating that, as with the second azide, the first azide also bridges the type 2 and type 3 sites.[3] In contrast, only azide A in ascorbate oxidase bridges these sites, and azides B and C bind to the type 3 and type 2 sites, respectively. In both systems, two open coordination sites for azide exist on the type 3 site and two positions are also exchangeable on the type 2 site. However, the differences in the coordination chemistry of laccase and ascorbate oxidase suggest that there is a distortion in the trinuclear site in ascorbate oxidase that prevents an additional azide from bridging the type 2 and type 3 sites, and instead two additional azides independently coordinate to each center. The distortion may involve steric hindrance, possibly from the protein side chain implicated in the slow binding kinetics of azide B or a misorientation of the open coordination positions on the type 2 and type 3 sites. The histidine residues that ligate the type 2 and type 3 coppers in ascorbate oxidase are conserved in *Neurospora crassa* laccase and human ceruloplasmin.[5,43] Although sequence data are not available for *Rhus* laccase, the homology observed among three other multicopper oxidases suggests that it is likely that the same histidine ligation set exists in *Rhus* laccase, and the differences in the coordination chemistry of *Rhus* laccase and ascorbate oxidase are not associated with differences in the identity of the type 2 or type 3 ligands. The differences may result from substitution of nonligand amino acids in the vicinity of the trinuclear site or from protein conformational differences that perturb the geometry of the ligation sphere of the trinuclear site.

The slower binding of azide B to ascorbate oxidase is not reflected in the dioxygen reactivity of the trinuclear site. In steady-state kinetic studies, the K_m for oxygen is 50 μM in ascorbate oxidase[44] and 21 μM in laccase,[45] indicating that affinity for oxygen is similar in the two systems. The rate constant for oxygen binding is not directly accessible in these enzymes. However, the turnover numbers have been measured and are 50000 min^{-1} for ascorbate oxidase[44] and 34000 min^{-1} for laccase.[45] These high turnover numbers indicate that oxygen binding must be substantially faster than the binding of azide B in ascorbate oxidase. Thus, the binding of azide B does not appear to be directly relevant to the binding mode of the physiological substrate, dioxygen (vide infra).

Our studies provide evidence for structural differences between the type 1 sites in laccase and ascorbate oxidase. In contrast, strong similarities are observed in the electronic and geometric structures of the type 2 sites in these enzymes. The reactivity of the trinuclear site in ascorbate oxidase with exogenous ligands exhibits interesting similarities and differences relative to our previous studies of laccase. Fluoride binds very similarly in the two enzymes. In addition, azide A binds as a bridging ligand between the type 2 and type 3 sites in ascorbate oxidase in a manner that parallels the binding of the second azide in laccase. However, two additional azide molecules coordinate terminally to the type 2 and type 3 coppers, respectively, whereas in laccase only a second azide binds to the fully oxidized enzyme and bridges the type 2 and type 3 sites. This difference indicates the presence of a distortion of the ascorbate oxidase trinuclear site that prevents an additional azide from bridging the type 2 and type 3 centers. The conservation of the type 2–type 3 bridging geometry between the two enzymes supports our earlier model that this binding mode may be active in the binding and multielectron reduction of dioxygen,[3,8] and it is apparent that only a single coordination position capable of bridged binding of exogenous ligands is required for efficient dioxygen reduction in the multicopper oxidases.

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Registry No. Cu, 7440-50-8; N_3^-, 14343-69-2; ascorbate oxidase, 9029-44-1; laccase, 80498-15-3; fluoride, 16984-48-8.

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